# METHOD AND APPARATUS FOR VIABLE AND NONVIABLE
# PROKARYOTIC AND EUKARYOTIC CELL QUANTITATION

## CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. Provisional Application No.
5    60/220,298, filed July 24, 2000.

## TECHNICAL FIELD

The present invention relates to methods for the rapid quantitation of both
viable and nonviable cells. More specifically, the invention involves incubating cells with
a metabolically activated visible fluorescent dye and measuring the fluorescence generated
10    by viable cells. Total cell populations (viable + nonviable) are separately determined by
measuring the native UV fluorescence of the cells. The two fluorescence readings are
directly related to the number of viable and nonviable cells. This permits the user to
determine the percent viability of a mixed population of live and dead cells.

## BACKGROUND OF THE INVENTION

15    The ability to quantify living cells is vitally important to the food, beverage,
pharmaceutical, environmental, manufacturing and clinical industries. Several methods are
currently employed by these industries to quantify prokaryotic and eukaryotic cells. These
methods include, but are not limited to, the standard plate count, dye reduction and
exclusion methods, electrometric techniques, microscopy, flow cytometry,
20    bioluminescence and turbidity.

The standard plate count permits the quantitation of living cells (or clumps
of cells) also known as colony forming units (cfu) when the cells are grown on the
appropriate medium under optimal growth conditions *(Microbial Ecology, Atlas, R.M. and
Bartha, R., Addison Wesley, Longman, New York, 1998)*. Current standards of viable
25    organism counts are often based on the standard plate count, particularly in the food
industry. However, colony counts are difficult to interpret since bacteria often clump or

form chains that can give rise to significantly inaccurate estimations of the total number of viable organisms in a sample. Also, bacteria, for example, can be in a "metabolically damaged" state and not form countable colonies on a given medium. This problem is more severe when selective media are used. Thus, the standard plate count does not provide a

5    definitive count of viable cells in a sample, which may be very important for certain purposes. Given these factors, such testing also requires skilled technicians who can distinguish individual colony forming units and who can aid in selecting appropriate growth medium. Moreover, the technique is not useful when rapid determination of cell counts is required since it often requires over 24 hours to obtain results.

10    Other tests, such as, dye reduction tests rely on the ability of cells to oxidize or reduce a particular dye (Harrington, 1998). Such methods are used to measure the activity of metabolically active organisms rather than provide a measure of the total number of viable cells in a sample. Dyes, such as methylene blue, coupled with microscopic counting, are routinely employed to determine the relative number of

15    microorganisms. The technique is widely employed but nevertheless suffers from factors that must be held constant during the assay, e.g., medium used, chemical conditions, temperature and the types of cells being examined. Also, dye reduction tests that incorporate microscopic counting techniques require trained technical personnel and often depend on subjective interpretations.

20    Dye exclusion methods of cell quantitation depend on the living cells having the ability to pump the dye out of the cell and into the surrounding fluid medium. While the dye may enter the interior of both living and dead cells, dead cells are not capable of actively pumping the dye out under the conditions normally used. Dye exclusion is commonly employed to enumerate animal, fungal and yeast cells. It is a method requiring

25    skill, correct timing and proper choice of dye. It is not applicable to certain microbes and it yields incorrect viable counts with stressed cells.

An accurate estimation of the number of viable yeast cells in a sample can be obtained by the slide viability technique (Gilliland, 1959). The yeast cells are suspended in a growth medium containing 6% gelatin and the suspension is placed in a

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hemocytometer slide. The cell suspension is incubated for approximately 20 hours and the numbers of micro colonies are counted. Cells that form micro colonies are viable and dead cells remain as single cells. This technique is considered by the brewing industry to be the most definitive test for counting the number of viable yeast cells. Unfortunately, the long

5    incubation time makes it unacceptable as a routine method.

Microscopic techniques typically involve counting a dilution of cells on a calibrated microscopic grid, such as a hemocytometer. A recent improvement in this technique is the direct epifluorescent filter technique (DEFT) (Pettipher et al, 1989). In this technique, samples are filtered through a membrane filter that traps the cells to be counted.

10    A fluorescent dye is attached to the cells, which are illuminated with ultraviolet light and counted. Unfortunately, the technique requires the use of an expensive microscope and a trained individual or an expensive automated system (Pettipher et al., 1989).

Yet other methods of quantitation use flow cytometry involves the differential fluorescent staining of cells suspended in a relatively clear fluid stream of low

15    viscosity. The cell suspension is mixed with the fluorescent dye and illuminated in a flow cell by a laser or other light source. The labeled cells are automatically detected with the use of a fluorescence detector focused on the cells (Brailsford and Gatley, 1993 and Pinder et al, 1993). The technique requires, and is limited by, expensive equipment. Some flow cytometric devices have been used by the food and dairy industry, but their application has

20    been limited by the high cost of instrumentation.

Bioluminescence has been routinely employed in the food sanitation industry to detect and quantify viable organisms and cells. The method involves the use of luciferin-luciferase to detect the presence of ATP (Harrington, 1998 and Griffith et al, 1994). When used to quantify cells, the technique depends on the assumption that there is

25    a constant amount of ATP in a living cell. ATP levels vary in a single cell over more than two orders of magnitude, making this method a relatively inaccurate technique for the enumeration of viable organisms in a sample.

Turbidity of a liquid sample can also be measured as an indication of the concentration of cells due to the light scattering and absorbing qualities of suspended cells

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(Harrington, 1998). The method is old but it is still employed to estimate the bacterial concentration in a sample. The method is rapid and simple but is highly inaccurate since all cells, particles and substances, including non-living particulate matter, interfere with the interpretation of the results.

5        The present invention for the quantitation of both viable and nonviable cells is designed to overcome at least five problems that have been identified within the field. First, the new technology circumvents the need for training personnel in how to plate, grow and count viable cells from colonies on agar plates. It also eliminates nearly all training and maintenance costs associated with most of the other methods. Second, the invention
10      substantially decreases the time needed to determine concentrations of cells such as yeast and bacteria. Under current methodologies, quantification requires from 24-72 hours (plate count and enrichment cultures), while the present invention permits accurate quantitation in less than 15 minutes. The methylene blue test is rapid; however, the accuracy is unacceptable for cultures that are less than 90% viable. The slide viability test is accurate
15      for large viability ranges but the time required for results is not suitable for routine use. Third, the new test is accurate over wide ranges of viability and has precision similar to the slide viability test. Fourth, the instant invention offers substantial cost savings over existing methods of cell quantitation. Fifth, the invention permits the simultaneous determination of both viable and total cells in a sample. This allows the user to accurately
20      establish the percent viability of a cell sample (the number of viable cells to total cells). Percent viability is a crucial measurement in many industries such as, the dairy and beer brewing industries and is currently carried out by the methylene blue test.

SUMMARY OF THE INVENTION

25      The present invention generally provides methods, kits, and devices for detecting and quantitating the number and/or percentage of viable cells in a sample. In one aspect the invention provides a method for determining the percent viability of cells in a sample, comprising providing a sample containing said cells, detecting the total cell count, contacting said cells with molecule or dye that is detectably altered by enzymatic activity

of a viable cell, detecting enzymatically altered dye or molecule, thereby detecting the number of viable cells and comparing the number of total cells with the number of viable cells thereby determining the percent viability.

In another aspect, a method for detecting viable cells is provided that comprises providing a sample containing cells, contacting said sample with a dye that diffuses or is transported into said cells and wherein said dye is detectably altered by enzymatic activity of a viable cell, thereby detecting viable cells in a sample.

Yet additional aspects of the present invention include methods for quantitating viable cells in a sample, comprising providing a sample containing said cells, contacting said cells with molecule or dye that is detectably altered by enzymatic activity of a viable cell, detecting ezymatically altered dye or molecule, thereby detecting the number of viable cells in said sample and obtaining a value therefrom and correlating the detected viable cell value with a standard value, thereby quantitating the viable cells in said sample.

Further aspects include methods for quantitating total and live cells in a sample, comprising measuring total fluorescence of cells in a sample and comparing to a standard value, thereby quantitating total cells in said sample; contacting a sample with a fluorescent dye that is metabolically altered by live cells; said dye having fluorescence properties that are measurably altered when modified by live cells, detecting the metabolic alteration of the dye thereby obtaining a measurement value and comparing said value to a standard value, thereby quantitating live cells in said sample.

Still other aspects of the present invention include methods for measuring the number of total and live yeast, bacteria or other cells in a sample, comprising measuring the native fluorescence of cells in suspension, contacting said cells with a dye that penetrates into the interior of yeast or bacteria and is metabolically modified to a measurable parameter by live cells, measuring the total fluorescence and fluorescence properties provided by the metabolic alteration of said sample and correlating said fluorescence to the number of total and live cells in said sample or a fraction of the sample and determining the percent viability of said sample.

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In certain embodiments the cells may be of any origin such as bacteria, yeast, or mammalian. In related embodiment the total cell count is determined by a method selected from the group consisting of native UV absorption, turbidity testing, hemacytometer measurements, fluorescence, and dye exclusion.

5      In yet other embodiments, the enzymatic activity that alters the dye or molecule is esterase activity. In further embodiments the enzymatically altered dye or molecule is fluorescein diacetate or OREGON GREEN™.

Other embodiments include measurement by a device such as, by a flurorometer.

10     The invention also provides kits for quantifying yeast or bacteria, comprising a cell suspension solution, a cell penetrating dye, and instructions for detecting dye that correlates to hemocytometer counts, plate counts or other methods of counting viable cells.

In certain embodiments the kit includes a dye that is enzymatically and

15    detectably altered following penetration of viable cells.

In certain aspects a kit for quantifying yeast or bacteria or mammalian cells is provided, comprising: a first container containing a first solution, a second solution containing a compound that penetrates cell membranes and is metabolized to a fluorescent dye or other detectable dye that is measurable, and instructions for using the same.

20     In other embodiments kits of the invention further comprise a means for mixing said first solution with a sample containing an unknown number of living cells and nonliving cells, means for concentrating the cells from the mixture of said first solution with said sample and removing solids from the remainder of said mixture, and measuring native fluorescence of cells in said solution.

25     In still yet other embodiments the kits may further comprise a means for mixing said second solution with said cells to form a second mixture, and means for illuminating the mixture of said second solution with said cells with excitation light and measuring fluorescence emitted by said mixture, and thereby determining the amount of

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metabolically modified dye present in the cells that is proportional to the number of viable cells in said second solution.

In other embodiments the kits may further comprise a third solution containing a compound or compounds that increase the rate of uptake of dye into cells or speeds up the rate of conversion the detectable fluorescent form of the dye inside said cells in second solution.

A device that comprises solid fluorescent material consisting of an adaptor and a compound that can be used to calibrate the instrumentation used for detecting fluorescence in the cells.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an example of solid calibration standards for an ultraviolet and visible wavelength fluorometer.

Figure 2 is a correlation chart of comparable Easy Count readings of total cell counts to total cell counts as determined by methlylene blue.

Figure 3 is a correlation chart of comparable Easy Count readings of viable cell counts to total cell counts as determined by methlylene blue.

Figure 4 is a plot of regression analysis demonstrating the relationship between the inventive method and those determined by methylene blue for total cell count.

Figure 5 is a plot of regression analysis demonstrating the relationship between the inventive method and those determined by methylene blue for viable cell counts.

Figure 6 is a linear correlation plot of hemacytometer counts vs. Easy Count Readings. Data points are the mean of three samples. The linear relationship is significant $P<.0001$.

Figure 7 is a linear correlation plot of hemacytometer counts that have been corrected for viability using methylene blue stain vs. Easy Count Readings. Data points are the mean of three samples. The linear relationship is significant P<.0001.

Figure 8 is a linear correlation between percent viability as measured by slide culture, and Easy Count values. Data points are the mean of three samples. The linear relationship is significant P<.0001.

Figure 9 is a plot representing fermentation tracking. Fermentation tracking in a laboratory fermentation using both the Easy Count and hemacytometer methods. Time 0 is the time that the cells were pitched into fresh wort. The Y-axes represent cell counts using a hemacytometer (squares) and active cells using the Easy Count (diamonds). Data points are the mean of three samples.

Figure 10 is a pot representing fermentation tracking during brewery scale fermentation. The Y-axes represent cell counts using a hemacytometer (squares) and the Easy Count (diamonds). Data points are the mean of three samples.

Figure 11 is a plot depicting Percent error between operators for the three methods is shown. Methylene Blue dead (stained) cells reported differences between operators of 28.3%, while Methylene Blue live (non-stained cells) was 21.0%. The error between operators for the Easy Count was significantly lower, at only 2.6%.

DETAILED DESCRIPTION

Briefly, the current invention describes novel methods that can be used to quantify live cells and total cells (total includes all cells in the sample, both viable and nonviable) such as yeast and bacteria. This allows the user to determine percent viability of the sample of cells. In one aspect, the instant invention comprises three steps: 1) Determination of total cells, 2) Determination of viable cells and 3) Calculation of percent viability. In certain embodiments the total cells are determined by washing and incubating the cells in a solution and then measuring the native UV fluorescence of the cells in a fluorometer permit determination of total cell populations. Subsequently the cells are

incubated with a compound that can be metabolically converted to a visible fluorescent dye such as fluorescein diacetate, coupled with an inducer of esterase activity such as dequalinium acetate, and then measuring the fluorescence thus permitting enumeration of viable cell populations. The fluorescent readings are correlated to standard counts such as

5 hemocytometer counts or to the slide viability counts. The two fluorescence readings are directly related to the number of total and viable cells respectively. This permits the user to calculate the percent viability of a mixed population of live and dead cells.

As those of ordinary skill in the art can readily appreciate the present invention may be modified in certain ways to achieve the same result. In brief, the present

10 invention utilizes one or more dyes or molecules that allow for the detection of all cells or total cells (e.g., yeast, bacteria, mammalian, etc.) in a sample and the same or different one or more dyes that is metabolized/derivatized by the viable cells in the sample to allow detection of the viable cells. Accordingly, the percent viability can then be readily determined. As can be appreciated adding substances such as detergent-like compounds,

15 surfactants, solvents, or other compounds that affect membrane polarity, membrane fluidity, permeability, potential gradient etc., to the sample to increase the rate at which the molecule or dye enters the cells in order to speed the process.

Other variations that are within the scope of the present invention include adding compounds that affect membrane polarity to decrease the rate of "leakage" of the

20 converted dye from the cells. Further, esterase enzyme inducing chemicals may be added to increase esterase activity in living cells such as naphthalene or dequalinium acetate or by environmental factors such as heat. In addition, compounds other than fluorescein diacetate, such as Calcein AM ,may also be used to detect metabolically active live cells.

The stability and shelf life of the fluorescein diacetate and other chemicals

25 may be increased by the addition of antioxidants or similar preservatives or by dissolving the FDA into other solvents besides acetone or by other stabilizing methods such as lyophilization. The fluorescence detection apparatus used may be designed for microscopic, surface, internal, solution and non-suspension sample formats.

Also included within the context of the present invention is software that permits the user to interface the fluorescence instrumentation to a computer for direct calculation of percent viability and cell concentration or other data processing or recording formats.

5        Addition of compounds to reduce background in the sample, such as hemoglobin may be utilized. Rinsing the sample in a buffer solution and centrifuging or filtering or otherwise retaining the cells as they are washed to remove any exogenous background fluorescence.

The differences between prokaryotes and eukaryotes may also utilized to
10      assist detection. For example, such easily detectable differences include cell membrane receptors, lack of organelles in prokaryotes, or metabolic differences. These differences can be utilized to distinguish between prokaryotes and eukaryotes by using dyes that penetrate only mitochondria or nuclei for example, or to take advantage of membrane and metabolic differences in these two cell types. This will allow the user to count a specific
15      prokaryote or a eukaryote in a mixture of cells that contains both types of cells. For example, one may determine if bacteria contaminate a yeast cell or blood cell population.

Other variations to the present invention include altering the pH of the reaction solutions to increase the sensitivity of the reaction. Changing concentrations of the solutes to increase or decrease the sensitivity of the reaction. Alterations to the solid
20      standards may be utilized to increase the sensitivity and dynamic range of the assay. Use of the viability assay to measure overall health or metabolic or growth status of the cells including ability to withstand stress. Use of the various steps of the tests independently, *e.g.*, to measure only total cells or only live cells.

Other methods of total cell determination may be made by using DNA
25      binding dyes, protein stains, cell membrane stains, antibody coupled stains, lipid dyes or other methods of detecting total cells in a sample. Viable cells may also be quantified using other methods that distinguish between live and dead cells such as surface markers, DNA stains, protein stains, antibody coupled stains, lipid dyes or other methods of detecting viable cells in a sample.

The solid standard can be made out of other materials such as, but not limited to, plastic, or by embedding chemicals such as fluorescein in a solid matrix of epoxy, acrylic, polyacrylamide or agarose or by coating a material like plastic with said chemical. Other chemicals, which have excitation and emission wavelengths in the range of the dyes or cells used to carry out the invention, could be used.

The instrument can be calibrated with solutions containing fluorescent chemicals such as fluorescein or OREGON GREEN™. Other configurations of the solid standard can be employed. The adaptor can be constructed to fit the type and make of instrument used to carry out the method. Different concentrations of the reagents may be used to carry out the method. Other methods of mixing and or concentrating samples may be used. The wash steps may also be eliminated, thus simplifying the procedure.

Yet further variations of the present invention include, but are not limited to, the use of an incubator to control the temperature of the dye conversion in the cells. Such incubation can take place in a plate counter or vial heater of some kind. Further, the samples may be arranged in an array format to allow high throughput detection.

The methods and kits of the present invention allow for the determination of the number of active cells by measuring the rate of conversion of dye by the cells. The number and activity of said cells may be determined without reaching the reaction endpoint.

The methodology has obvious application in determining the activity of yeast or bacteria in industrial fermentation applications. Thus, the methods and kits can be used to predict the number of cells required to carry out fermentation based on viable cells rather than on total cells.

As those of ordinary skill in the art can readily appreciate, the instant invention can be carried out in a single vessel and solution.

The instant invention also has applicability in assessing the activity, vitality, or number of cells under various storage conditions, comparing the metabolic activity of different cells, developing pitching rate charts for fermentation applications, and use of the method as a self-contained laboratory.

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The present invention provides methods, kits and apparatuses for simple dye associated quantitation that allows one to inexpensively determine total cell counts and viable cell counts in a particular sample. An individual of ordinary skill in the art will readily appreciate that alternatives to the steps herein described for quantitating cells may

5    be used and are encompassed herein. Accordingly, all alternatives will use a kit or method wherein a dye is utilized to stain cells and a method is used to detect or quantify the dye. One key aspect of this invention is its ability to simultaneously determine total cells and live cells in a sample in short times compared to standard methods. In preferred embodiments, detection is completed in less than 4 hours, in others in less then about 3

10   hours, and yet further, in less than about 2 hours, while in specific embodiments, detection is completed in less than about 1 hour, less then about 45 minutes, less than about 30 minutes, less than about 15 minutes, and less than about 10 minutes at low cost.

All patents, patent applications and references cited herein are incorporated in their entirety. Accordingly, incorporated herein by reference are U.S. Patent Numbers:

15   5437980; 5563070; 5582984; 5658751; 5436134; 5939282; 4783401; 3586859

# EXAMPLES

20   ## EXAMPLE I
### Materials and Calibration

The following shows examples of solutions, volumes and concentrations that can be used to carry out the invention. Other concentrations and volumes and different

25   buffers with different pH values may be used. The samples and reaction solutions may be provided in any volume necessary for detection, however, in the present embodiment, small volumes (less than 1 ml) are utilized such that reagents and sample amounts are kept to a minimum. The volumes and concentrations to be utilized are any that are convenient

to the practitioner of the methodology. In certain embodiments, the sample and reagent amounts range from 1 to 10,000 micro liters.

The reader used in the present embodiment is a "picofluor" hand-held fluorometer from Turner Designs, Sunnyvale, CA 94086. Any fluorometer that can
5    measure fluorescence at specific wavelengths for detection of the dyes or cells can be employed in the invention. Ideally, the fluorometer can switch back and forth from visible (486 nm excitation with a 10 nm bandwidth and 550 emission with a 10 nm bandwidth) and UV (300-400nm excitation and 410-700nm emission) modes without changing filters or making other adjustments; however, this is not necessary to carry out the methodology.
10   Other wavelengths may be used in combination with different dye types or cell types. The wavelength chosen will be dependent on the dye or cell type chosen for staining.

The instrument needs to be calibrated in a range that permits quantitation of cells. Calibration can be carried out with solutions such as fluorescein or Oregon green or with the use of solid standards as described below.

15   Materials

**Solid Calibration Standards:**

Materials

Colored glass rods: Mint green for the visible mode calibration, and translucent

blue for the ultraviolet (UV) mode calibration.

20   Solid calibration adapter: Black diacetyl plastic machined to fit the instrument.

Glass rods are glued into the solid calibration adapter and sealed with a plastic cap,

(See Figure 1).

**Solutions A (Cell Preparation): 1X PBS**

25   Ingredients (for 10X stock solution)

80 grams NaCl

2.0 grams KCl

14.4 grams $Na_2HPO_4$

2.4 grams $KH_2PO_4$

NaOH (enough to reach a PH of 7.4)

This recipe makes a 10X stock solution. It must be diluted 1:10 into distilled water to make a 1X working solution before being used.

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**Solution B (Live Cell Suspension):**

Ingredients: 0.0275 grams dequalinium acetate in

30 mL 10X Sodium acetate buffer: (13.6 grams Sodium acetate in 100mL $ddH_2o$)

10 **Stock Solution C (Live Cell Reaction Stock):**

Ingredients:

Fluorescein diacetate (FDA), 30mg/10mL in Acetone

Protocol

I.    Calibration using solid standards

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**To calibrate the instrument in "A" mode (UV): Standard Value = 500**

1)    Remove the mini cell receptacle from the instrument.

2)    Be sure that the instrument is in "A" mode; the letters UV should appear in the lower left corner of the screen.

20    3)    If the instrument is not in "A" mode, press the **A/B** key on the instrument keypad.

4)    Press the **CAL** key on the keypad; when prompted, press **ENTER** to continue.

5)    When asked to insert the blank, insert the solid standard labeled "A"

25    into the instrument with the letter "A" facing down and to the right.

6)    When asked to insert the "cal", insert the solid standard labeled "A" into the instrument so that the letter A is facing towards you, and the white cap is on top.

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**To calibrate the instrument in "B" mode (Visible): Standard Value =500**

1)     Follow all the instructions for calibration in "A" mode, making sure that that the instrument is now in "B" mode, and that the solid standard labeled "B" is now being used.

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## EXAMPLE II

10     ## Total Cell Counts

Total cell counts can be determined by a variety of methodologies, including using the fluorometer noted above. UV operation mode is selected. A fixed volume (200 microliters) of **Cell preparation solution** is added to the glass sample vial. 5 microliters

15     of sample (yeast cells) is then added to the **Cell preparation solution** in the vial and centrifuged for about 30 seconds to sediment the a cell pellet. The cell pellet is resuspended in about 100 microliters of **Cell preparation solution** to the sample vial. The sample vial is then read in fluorometer. See Figure 2.

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## EXAMPLE III

### Viable Cell Counts

Live cells are quantitated utilizing a dye that is detectably altered by an

25     intracellular enzyme. For example, the fluorometer noted above is set in visible mode and a fixed volume (200 microliters) of **Cell preparation solution** is added to the sample vial. 5 microliters of sample is added to the solution A in the sample vial and centrifuged for 30 seconds. 100 microliters of **Suspension solution** is added to the sample vial and 5 microliters of **Reaction solution** is added to the sample vial. The sample is mixed and

placed in the reader and fluorescence determined at time zero and again at 15 minutes. This is the value that will be compared to the Easy Count correlation chart for conversion to cells/ml. See Figure 3.

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## EXAMPLE IV
### Yeast Quantitation

Yeast performance is critical to the development of quality beer. For this reason, methods of yeast analysis are an important element of the brewing process.
10 Traditional methods including hemacytometer counting and methylene blue staining are rapid, but inaccurate and unreliable. Slide culture is an accurate measure of yeast viability, but requires a lengthy incubation period of 18 to 24 hours. As an alternative, the fluorometric assay, described above is based on the metabolic activity of the yeast culture to provide brewers with a rapid and accurate estimation of active cell number. This method
15 was compared to the hemacytometer counting technique as an estimation of cell number, and to both methylene blue staining and slide culture as measures of vitality and prediction of fermentation performance. The inventive method correlated to the hemacytometer, methylene blue, and slide culture with $R^2$ values of .985, .987, and .962 respectively, P<.0001. An error analysis was carried out by the inventive methods, hemacytometer and
20 methylene blue staining techniques for multiple operators performing the tests. Thus, the present invention could be used to determine correct pitching rates, monitor fermentation and propagation, and for other applications involving cell quantitation.

25 Yeast

All yeast cultures were obtained from Wyeast Laboratories, Mt. Hood, OR. Yeast samples for the experiments comparing the hemacytometer, methylene blue staining, and slide culture to the inventive methods were a 1084 strain of *Saccharomyces cerevisiae*. Yeast cultures tested during laboratory scale fermentations were strain 1968. Brewery
30 scale fermentations were performed using yeast strain 1056.

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### Hemacytometer Counts

Hemacytometer counts were performed according to the ASBC method (6,8). Samples were removed from a slurry with an initial concentration of 198 million

5 cells per ml, as determined by hemacytometer count, and diluted in spent wort to maintain cell integrity. Each dilution was counted in the hemacytometer as well as measured using the present method. Experiments were carried out in triplicate.

### Methylene Blue Staining

10 Methylene blue staining was performed according to the ASBC method (6,8). Samples were removed from a slurry with an initial concentration of 198 million cells per ml, as determined by hemacytometer count, and diluted in spent wort to maintain cell integrity. Each dilution was stained and counted in a hemacytometer, as well as measured using the present method. Hemacytometer counts were corrected for viability

15 according to the staining results. Experiments were carried out in triplicate.

### Slide Culture

Slide culture was performed according to a modified version of the protocol for preparation of slide cultures for the examination of yeast and mold (5). Ten ml of yeast

20 strain 1028 at a concentration of 433 million cells per milliliter, as determined by a hemacytometer count, were placed into a 43 degree Celsius water bath. Aliquots were removed at time intervals 0, 2, 4, 6, 8, 10, 15, 20, 30, and 40 minutes. Each was tested using the inventive method. Measurements were taken in triplicate and averaged. Slide culture samples were diluted 1:100 into wort containing 6% gelatin. 10µl of the sample

25 were then placed on a micro slide, covered and sealed with petroleum jelly. Each slide was incubated for 20 hours at 18 degrees Celsius before microscopic examination (Microscope model, Leica DMLB). Viability was determined with the assumption that living cells had formed micro colonies, while nonviable cells remained single.

<u>Determination of Active Cell Number Using the Instant Invention</u>

The inventive method for determining total active cell number is based on the metabolic activity of the yeast culture. The technology involves exposing cells to proprietary chemicals that enter cells through diffusion. These molecules are converted to a

5    fluorescent form by metabolically active cells. This fluorescent signal is quantified in a handheld battery operated fluorometer model GP320 GenPrime Inc, Spokane, WA. The protocol is as follows; 50µl of yeast sample was added to 500µl of cell prep solution in a 1ml glass test cuvette. 50µl of dye solution was added; the cuvette was capped, and incubated for 5 minutes. After incubation, the cuvette was shaken, and the fluorescent

10   signal quantitated in the GP320. These values were compared to the hemacytometer and methylene blue staining methods by performing tests with the inventive method on the diluted samples from these experiments. Readings were taken in triplicate and averaged. These relationships were analyzed by linear regression using Statview, SAS institute, Cary NC.

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<u>Fermentation Tracking</u>

*Laboratory Scale:* Laboratory scale fermentation tracking was carried out in a 300ml flask by inoculating 150ml wort with 5ml yeast strain 1968, with an initial concentration of 420 million cells per ml, and monitoring growth using a hemacytometer

20   and the inventive methods. Cells were grown at room temperature (21°C). Samples were taken every 45 minutes for 5.25 hours and then periodically over the next 48 hours.

*Brewery Scale:* Brewery Scale fermentation tracking was carried out during a typical fermentation cycle, at the Steam Plant Grill, Spokane, WA 99201. Hemacytometer counts and corresponding readings using the instant invention were made

25   daily for 13 days beginning immediately following pitching.

<u>Error</u>

Percent error between operators was determined for the inventive method "Easy Count" method, hemacytometer counts, and the methylene blue staining method. Error analysis was performed using Microsoft Excel.

*Hemacytometer:* Three operators performed hemacytometer analysis of a
5   yeast strain 1028 slurry according to the ASBC method. Each operator prepared and measured 15 samples. Results were averaged for each operator, and error between operators was calculated.

*Methylene Blue:* The 15 hemacytometer samples from above were stained with methylene blue according to the ASBC method. Each of the three operators counted
10  stained cells for each sample. Results were averaged for each operator, and error between operators was calculated.

*Easy Count:* Easy Count tests were performed on 15 replicate samples by each of the three operators. Results were averaged for each operator, and error between operators was calculated.
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### Hemacytometer Counts

Figure 6 shows the correlation between the Easy Count values and the cells/ml results of the hemacytometer. A statistically linear relationship was found between cell counts obtained by the ASBC standard method of microscopic examination
20  using a hemacytometer and values obtained using the Easy Count, $R^2=0.985$.
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### Methylene Blue Staining

Figure 7 illustrates the linear correlation found between the Easy Count
25  method and the ASBC method for methylene blue staining. A statistically linear relationship was found between the Easy Count, and hemacytometer counts corrected for viability, $R^2=0.987$
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These results suggest that the Easy Count can be used to accurately predict active cell number. Using the results of the correlation, it is possible for the brewer to accurately determine the correct pitching rate using the Easy Count method based on 1 million active cells per ml per degree plato of wort. Additionally, the method can be used

5 to monitor fermentation, propagation, and for other applications involving the quantitation of cells.

Slide Culture

A linear relationship was found between the Easy Count and slide culture for yeast viability, as shown in Figure 8.

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The correlation to slide culture confirms that the Easy Count only measures active cells, since the total number of cells in this experiment remains constant.

15 Fermentation Tracking

Cell growth was measured during laboratory and brewery scale fermentations using both the ASBC method for hemacytometer counts, and the Easy Count method. Figure 9 shows cell growth tracked by both methods during laboratory scale fermentation. Figure 10 is an example of a brewery scale fermentation tracked by both

20 methods.

Error

Results from the experiments were averaged for each operator as shown in

25 Table 1. Percent error between operators was calculated by dividing the standard deviation of the mean by the mean, and multiplying the result by 100. Easy Count reported significantly lower error between operators than the other methods. These results are graphed in Figure 11.

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Table I

| Data in Millions of cells/ml | Easy Count mean | Methylene Blue live mean | Methylene Blue dead mean |
|---|---|---|---|
| **Operator 1** | 195.9 | 158.6 | 27.9 |
| **Operator 2** | 197 | 122.3 | 23.6 |
| **Operator 3** | 187.7 | 187.7 | 40.3 |
| | | | |
| Mean | 193.5 | 156.2 | 30.6 |
| Std. Dev. | 5.1 | 32.8 | 8.7 |
| %Error | 2.6 | 21.0 | 28.3 |

Results of the error experiments confirm previous research reporting the inaccuracies of hemacytometer counts and methylene blue staining (1,2,3,4,7). The low error associated with the Easy Count method is an improvement on these traditional techniques.

Percent error is of particular importance to the brewer due to the exacerbation of inaccuracies in the calculation of cells/ml. For example, when calculating cells/ml from a hemacytometer count of 180 live cells, and 15 dead cells, (counting all 25 fields and using a 1:100 dilution) the result would be 180 million live cells/ml (180*100*10000) and 15 million dead cells/ml (15*100*10000). If the error between operators when performing the live cell test is 21%, then the live cell result could be between 142-218 million cells/ml, a difference of 76 million cells/ml. With a percent error of 28% between operators, the dead cell result could be between 11-19 million cells/ml. This could result in reported viabilities between 87% and 96% for the same sample. The Easy Count has much less error associated with its performance. A reading of 6000 in the Easy Count would be 197 million active cells/ml (see equation generated in Figure 7.). A percent error of 3% between operators gives a range between 191-203 million cells/ml, a difference of only 12 million cells/ml. The very low error associated with the performance of the Easy Count provides much more reliable information to the brewer.

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